Identification and Expression Profile of Multiple Genes in Response to Magnesium Exposure in *Culex quinquefasciatus* Larvae

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J. Med. Entomol. 47(6): 1053–1061 (2010); DOI: 10.1603/ME10028

ABSTRACT Magnesium is crucial for baculovirus transmission in *Culex nigripalpus* (Theobald) and *Culex quinquefasciatus* (Say) larvae. However, the mechanistic role of magnesium in baculovirus transmission is unknown. To investigate the possible role of host response factors in baculovirus transmission, suppression subtractive hybridization was used to identify genes differentially transcribed after magnesium exposure in *Cx. quinquefasciatus* larvae. Suppression subtractive hybridization was performed in both directions enriching for cDNAs differentially transcribed between a nonmagnesium larval control and magnesium (15 mM $MgSO_4$) treatment of *Cx. quinquefasciatus* larvae held for 1 h at 27°C. Clones from differentially transcribed genes were evaluated by sequencing, and relative gene transcription levels were analyzed using quantitative real-time polymerase chain reaction quantitative real-time polymerase chain reaction. Target transcripts up/downregulated by magnesium included *Cx. quinquefasciatus* troponin C, isocitrate dehydrogenase, allergen, cytochrome b5, chymotrypsinogen, apolipophorins, tryptase gamma, carboxylesterase, prolylcarboxypeptidase, imaginal disc growth factor, aldehyde dehydrogenase, tropomyosin-1, chitotriosidase, heat shock protein 70 B2, inorganic phosphate cotransporter, and many other hypothetical protein genes. Magnesium can alter gene transcription in a vector mosquito population, and understanding this process can provide insight into the mechanistic role of magnesium in baculovirus transmission.

KEY WORDS magnesium, *Culex quinquefasciatus*, gene transcription, suppression subtractive hybridization

The critical role of divalent ions in the larval medium for the mediation (Mg^{2+}) and inhibition (Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}) of *Culex nigripalpus* (Theobald) deltabaculovirus (CuniNPV) infection in *Culex* larvae has been reported (Becnel et al. 2001). Infections of CuniNPV are restricted to nuclei of midgut epithelium, with mortality occurring 72–96 h postinfection. Magnesium is only required for the initial stage of the infection process before the virions entering the midgut cells (Becnel et al. 2001). The high infectivity and pathogenicity of CuniNPV against the principal vectors of West Nile virus in North America make CuniNPV an attractive candidate for future development as a biopesticide (Becnel 2006). Metal-induced gene expression in the mosquito midgut has been previously documented: exposing *Aedes aegypti* (L.) larvae to heavy metals showed AEIMUC1 (*Ae. aegypti* intestinal mucin) to be a metal-responsive gene, MRG (Rayms-Keller et al. 2000), and molecular biological studies have identified midgut genes induced by heavy metals in the midgut epithelium of aquatic arthropods (Beaty et al. 2002).

In the current study, suppression subtractive hybridization (SSH) is used to identify genes that are differentially transcribed during magnesium treatment of larval *Culex quinquefasciatus* (Say). Expression patterns are confirmed by quantitative real-time polymerase chain reaction (qPCR), and the regulated genes that were identified are discussed. In addition, a time course study of gene transcription in response to Mg^{2+} in *Culex* larvae was also examined for known genes. Characterization of genes induced by Mg^{2+} in *Culex* larvae could provide a mechanistic understanding of gene expression in larvae exposed to heavy metals.

Materials and Methods

RNA Extraction. *Cx. quinquefasciatus* (Orlando, FL strain, maintained since 1952) were reared in the insectary of the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology, United States Department of Agriculture–Agricultural Research Service (Gainesville, FL) following previously established protocols (Gerberg et

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Identification and Expression Profile of Multiple Genes in Response to Magnesium Exposure in Culex quinquefasciatus Larvae	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Department of Agriculture,Mid South Area-Agricultural Research Service, Biological Control of Pests Resear,59 Lee Road,Stoneville,MS,38776**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Magnesium is crucial for baculovirus transmission in Culex nigripalpus (Theobald) and Culex quinquefasciatus (Say) larvae. However, the mechanistic role of magnesium in baculovirus transmission is unknown. To investigate the possible role of host response factors in baculovirus transmission, suppression subtractive hybridization was used to identify genes differentially transcribed after magnesium exposure in Cx. quinquefasciatus larvae. Suppression subtractive hybridization was performed in both directions enriching for cDNAs differentially transcribed between a nonmagnesium larval control and magnesium (15mMMgSO4) treatment of Cx. quinquefasciatus larvae held for 1 h at 27 C. Clones from differentially transcribed genes were evaluated by sequencing, and relative gene transcription levels were analyzed using quantitative real-time polymerase chain reaction quantitative real-time polymerase chain reaction. Target transcripts up/downregulated by magnesium included Cx. quinquefasciatus troponin C, isocitrate dehydrogenase, allergen, cytochrome b5 chymotrypsinogen, apolipophorins, tryptase gamma, carboxylesterase, prolylcarboxypeptidase, imaginal disc growth factor, aldehyde dehydrogenase, tropomyosin-1, chitotriosidase, heat shock protein 70 B2, inorganic phosphate cotransporter, and many other hypothetical protein genes. Magnesium can alter gene transcription in a vector mosquito population, and understanding this process can provide insight into the mechanistic role of magnesium in baculovirus transmission.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**9**	

al. 1994). Magnesium treatment (15 mM $MgSO_4$) of third instar *Cx. quinquefasciatus* larvae (250–300) was carried out for 1 h at 27°C for the polymerase chain reaction (PCR)-select cDNA subtraction. This magnesium concentration has been shown to be optimal for CuniNPV transmission (Becnel et al. 2001). Control *Cx. quinquefasciatus* larvae (250–300) were collected at room temperature (27°C). The total RNAs were extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was isolated applying Oligotex-dT suspension (Qiagen, Valencia, CA). RNA and mRNA samples were quantified by SmartSpec Plus spectrophotometry (Bio-Rad, Hercules, CA).

Subtractive Hybridization. Differentially expressed mRNA populations, both magnesium (Mg^{2+}) treatment and control, were converted into cDNA using the PCR-Select cDNA Subtraction Kit (BD Clontech, Roche Molecular Systems, Alameda, CA). Subtractive cloning was performed, as previously described, and differentially expressed cDNA sequences were used to construct a subtracted cDNA library (Patel and Sive 2001, Zhao et al. 2009). In the generalized subtraction scheme, the mosquitoes to be compared are the [+] or tracer (Mg^{2+} treatment) and the [−] or driver (non-Mg^{2+} treatment, control), in which mRNAs expressed in the tracer and not the driver are isolated. Hybrids that form include sequences common to both mosquito populations. The unhybridized fraction is enriched for sequences that are preferentially expressed in the tracer mosquito population. Differentially expressed cDNA sequences were used to construct a subtracted cDNA library for further study.

PCR-Select cDNA Subtraction Library. Forward and reversed subtracted libraries were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA). Transformed plasmids were inserted into One Shot TOP10 Competent Cells (Invitrogen) and grown overnight on Luria-Bertani (LB) plates containing ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). When the cell transformation was conducted, the cells were incubated in the 37°C shaker for exactly 1 h, and plated out in the LB + ampcillin + X-gal plates. In that case, we limited the clone number in the plate (slightly over 100) so as they were not overly crowded. We picked out almost all clones on the plates. For each library, >100 white colonies were isolated and grown overnight in LB-ampicillin broth at 37°C and 235 rpm in the Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ).

Gene Sequencing of PCR-Select cDNA Subtraction Library. Clones of the subtracted library were purified with QIAprep Minipreps (Qiagen, Valencia, CA). The 0.5 μg of plasmid DNAs was then digested by using 2.5 U of *Eco*RI enzyme for 1.5 h, and was run on a 1% agarose gel to confirm the DNA insert. Selected clones were then sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida (Gainesville, FL). The ABI Big Dye terminator sequencing was used for DNA sequencing. The sequences were analyzed using the National Center for Biotechnology Information BLASTN program to identify sequence homologies. The homologous DNA fragments in the cDNA or mRNA were also recorded in Table 1. Hypothetical and conserved hypothetical proteins were further analyzed using the ExPASy Web site (http://ca.expasy.org) "DAS" Transmembrane Prediction Server to predict potential transmembrane segments.

cDNA Synthesis for qPCR. A 3-μg aliquot of purified RNA (from ≈100 larvae per sample) was reverse transcribed in a 20-μl reaction volume using Clone AMV First-Strand Synthesis Kit and using oligo$(dT)_{20}$ primer for cDNA synthesis, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). To ensure that no genomic DNA contaminated the sample, we used only oligo(dT) primer to synthesize the first-strand cDNA library for the reverse transcription PCR. The reaction was terminated by heat inactivation at 95°C for 5 min. The cDNA samples for qPCR from Mg^{2+} treatment and control were diluted by adding 80 μl of ddH_2O (300 ng/μl) and stored at −20°C.

To design gene-specific primers, detailed analyses of the nucleotide sequence of genes found in the library were performed using the PRIMER3-Design Primer Pairs and Probes program from Biology Workbench (http://workbench.sdsc.edu). The primers for the *Cx. quinquefasciatus* actin gene (GenBank accession number: XM 001866828) were also designed for internal control and comparison. The primers are listed in Table 2.

qPCR Amplification. The qPCR assay for *Culex* genes was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a volume of 15 μl on an Applied Biosystems 7300 Fast Real-Time PCR System (Foster City, CA). The PCR mixture consisted of 1 μl of diluted cDNA (300 ng/μl), 0.5 μM primers, and 1× master mix. In every qPCR run, actin was used as an internal control to normalize for variation in the amount of cDNA template. The PCR primers for actin gene were CxActin629-F 5′-TGCGTGACATCAAGGAGAAG-3′ and CxActin-893R 5′-GTGTTGGCGTACAGGTCCTT-3′. The PCR thermal cycling parameters were the same as previously described (Zhao et al. 2008, 2009). Relative expression levels were calculated as follows. First, *CxGENE* transcript levels relative to a standard (*CxACTIN*) were calculated using the formula Δ cycle threshold (CT) = CT (*CxGENE*) − CT (*CxACTIN*). Second, an average ΔCT value for each sample was calculated. Third, relative expression levels were calculated using the equation $100 \times 2[-\text{average } \Delta CT]$. Lastly, fold increases or decreases were calculated using Mg^{2+} treatment RNA relative expression level/non-Mg^{2+} treatment control RNA relative expression level. The detailed analyses of the nucleotide sequence of all up/downregulated genes were compared between Mg^{2+} treatment and control. When the relative gene expression was >1, the gene expression of treatment was upregulated. However, when the relative gene expression was <1, the gene expression of treatment was downregulated.

Table 1. Genes identified from *Culex quinquefasciatus* larvae in response to magnesium exposure

Clone	Accession no., organism, putative identity, mRNA/cDNA length	Gene regions
Known proteins		
1AB-3, 1AB-16, 1AB-53	ref XM_001844392.1 *Cx. quinquefasciatus* troponin C partial mRNA, length = 1,421	nt: 1146–767, nt: 1187–720
1AB-5	ref XM_001844978.1 *Cx. quinquefasciatus* isocitrate dehydrogenase partial mRNA, length = 1,303	nt: 504-1057
1AB-14, 1AB-35, 1B-9	ref XM_001869433.1 *Cx. quinquefasciatus* allergen partial mRNA, length = 911	nt: 911–395, nt: 239–878
1AB-18	ref XM_001863979.1 *Cx. quinquefasciatus* cytochrome b5 partial mRNA, length = 554	nt: 281–113
1AB-19, 1B-6	ref XM_001861639.1 *Cx. quinquefasciatus* chymotrypsinogen partial mRNA, length = 801	nt: 801–535
1AB-25	ref XM_001849258.1 *Cx. quinquefasciatus* apolipophorins partial mRNA, length = 10,169	nt: 10122–9807
1AB-36	ref XM_001847441.1 *Cx. quinquefasciatus* tryptase gamma partial mRNA, length = 828	nt: 75–381
1AB-42	ref XM_001868370.1 *Cx. quinquefasciatus* carboxylesterase partial mRNA, length = 1,695	nt: 321–493
1AB-51	ref XM_001850434.1 *Cx. quinquefasciatus* prolylcarboxypeptidase partial mRNA, length = 1,506	nt: 1328–1506
1B-4	ref XM_001844621.1 *Cx. quinquefasciatus* imaginal disc growth factor, mRNA, length = 1,384	nt: 435–733
1B-8, 1B-17	ref XM_001845150.1 *Cx. quinquefasciatus* aldehyde dehydrogenase, mRNA, length = 1,499	nt: 927–1274
1B-10	ref XM_001841858.1 *Cx. quinquefasciatus* isocitrate dehydrogenase cytoplasmic, mRNA, length = 1,407	nt: 951–1290
1B-20	ref XM_001864200.1 *Cx. quinquefasciatus* tropomyosin-1, mRNA, length = 875	nt: 875–827
1B-24	ref XM_001867666.1 *Cx. quinquefasciatus* chitotriosidase-1, mRNA, length = 2,643	nt: 2484–2217
1B-29	ref XM_001861401.1 *Cx. quinquefasciatus* heat shock protein 70 B2, mRNA, length = 1,917	nt: 821–1287
5B-7	ref XM_001847074.1 *Cx. quinquefasciatus* inorganic phosphate cotransporter, mRNA, length = 1,569	nt: 177–1
Hypothetical proteins		
1AB-4	ref XM_001851302.1 *Cx. quinquefasciatus* hypothetical protein partial mRNA, length = 1,068	nt: 1040–495
1AB-15	ref XM_001858971.1 *Cx. quinquefasciatus* hypothetical protein partial mRNA, length = 1,311	nt: 300–1
1AB-29	ref XM_001862348.1 *Cx. quinquefasciatus* hypothetical protein partial mRNA, length = 827	nt: 639–127
1AB-34	ref XM_001847371.1 *Cx. quinquefasciatus* hypothetical protein partial mRNA, length = 558	nt: 270–498
1AB-44	ref XM_001850204.1 *Cx. quinquefasciatus* hypothetical protein partial mRNA, length = 2,078	nt: 735–858
1B-25	ref XM_001847210.1 *Cx. quinquefasciatus* hypothetical protein, mRNA, length = 2,000	nt: 1269–1711
Conserved hypothetical proteins		
1B-7, 1B-14	ref XM_001866472.1 *Cx. quinquefasciatus* conserved hypothetical protein, mRNA, length = 747	nt: 510–708
1B-21	ref XM_001851203.1 *Cx. quinquefasciatus* conserved hypothetical protein, mRNA, length = 657	nt: 1–102
1B-22	ref XM_001859612.1 *Cx. quinquefasciatus* conserved hypothetical protein, mRNA, length = 612	nt: 612–292
1B-32	ref XM_001848166.1 *Cx. quinquefasciatus* conserved hypothetical protein, mRNA, length = 1,998	nt: 1830–1993
5B-5	ref XM_001861615.1 *Cx. quinquefasciatus* conserved hypothetical protein, mRNA, length = 691	nt: 685–501
Ribosomal proteins		
1AB-8, 1AB-9, 1B-53, 1AB-52	ref XM_001864716.1 *Cx. quinquefasciatus* ribosomal protein S25 partial mRNA, length = 566	nt: 159–490, nt: 490–30, nt: 32–490
1AB-11, 1AB-40	gb DQ401444.1 *Cx. quinquefasciatus* clone CpiLD-A01 large subunit ribosomal RNA, partial sequence; mitochondrial, length = 231	nt: 231–61
1AB-21	ref XM_001861287.1 *Cx. quinquefasciatus* 60S ribosomal protein L14 partial mRNA, length = 660	nt: 373–602
1AB-24	ref XM_001848606.1 *Cx. quinquefasciatus* 60S ribosomal protein L3 partial mRNA, length = 1,423	nt: 945–393
1AB-32, 1B-12	ref XM_001846289.1 *Cx. quinquefasciatus* 60S ribosomal protein L29 partial mRNA, length = 455	nt: 18–349
1AB-49	ref XM_001842837.1 *Cx. quinquefasciatus* 60S ribosomal protein L32 partial mRNA, length = 613	nt: 218–27
1B-13, 1B-28	ref XM_001841875.1 *Cx. quinquefasciatus* 60S ribosomal protein L8, mRNA, length = 998	nt: 636–801
1B-23	ref XM_001845506.1 *Cx. quinquefasciatus* 60S ribosomal protein L4, mRNA, length = 1,477	nt: 579–274
1B-27	ref XM_001863381.1 *Cx. quinquefasciatus* ribosomal protein L11, mRNA, length = 815	nt: 25–428

Time Course Study. Magnesium treatment (15 mM $MgSO_4$) of third instar *Cx. quinquefasciatus* larvae was carried out for 15 min, 1 h, 2 h, and 4 h at 27°C (≈250 larvae each time point) to trace the RNA expression level of differentially transcribed genes as identified from SSH. Control *Cx. quinquefasciatus* larvae (≈250 larvae each time point) were simultaneously collected without magnesium treatment at room temperature (27°C). The time course study was replicated three times. The RNA was extracted and cDNA was created, as previously described in this study, and qPCR was performed using primers designed from genes found by SSH (Tables 1 and 2).

Statistical Analysis. Comparisons of means were analyzed using Student's *t* test, and *t* values and *P* values were reported when normality and equal variance tests were passed. Significant differences between the data were determined using SigmaPlot software (SigmaPlot11.2, Systat Software, San Jose, CA).

Results

Identification of Genes Specifically Transcribed During Magnesium Treatment in *Cx. quinquefasciatus*. To reveal how larval genes were specifically transcribed during magnesium exposure in *Cx. quinquefasciatus*, we used subtractive hybridization to construct a subtraction cDNA library from control larvae and larvae that had been exposed to magnesium for 1 h. Clones of differentially transcribed genes were examined by sequencing. Using National Center for Biotechnology Information blast to analyze the sequences, we found 22 clones encoding *Cx. quinquefasciatus* known genes representing 15

Table 2. Primers used for quantitative RT-PCR

Primer name[a] sequence
1AB-3R 5′-ATGATGACCGGTTAAGCTGC-3′
1AB-3F 5′-AAAGGCGAGGGACTTTCTTC-3′
1AB-4R 5′-GACACCCAACAAAAACACCC-3′
1AB-4F 5′-TTGGTTGGTTGGGAGAATGT-3′
1AB-11R 5′-CGACCTCGATGTTGGATTAAG-3′
1AB-11F 5′-AGAAACCAACCTGGCTTACG-3′
1AB-15R 5′-GGTCCTGAAACACTCGGAGA-3′
1AB-15F 5′-GAAGTTTCGAGGTCCCTTCC-3′
1AB-18R 5′-GTGAGGAGGTGCTGGTTGA-3′
1AB-18F 5′-AGGCCGCTTTCTTCATTTTT-3′
1AB-21R 5′-CCCTCGCCTTAACCCTTAAC-3′
1AB-21F 5′-GAAGAAGGACGGCAAGAAGA-3′
1AB-24R 5′-ATCGACCATCTCGTCCTGTC-3′
1AB-24F 5′-CAGATCCGTCTGATCAAGCA-3′
1AB-25R 5′-GCGCCAAGGGATAAACTGTA-3′
1AB-25F 5′-TAATGGCGTGTTGGATGATG-3′
1AB-29R 5′-GAGTCAGTGAGCGAGGAAGC-3′
1AB-29F 5′-TATCCGCTCACAATTCCACA-3′
1AB-34R 5′-GGACATCTTCTTGAAGGCCA-3′
1AB-34F 5′-GTACGCCAAGAACCCCAAC-3′
1AB-36R 5′-TCAATGCGATCGATGGTAAA-3′
1AB-36F 5′-AGCCTCGGTTATCTCGTTGA-3′
1AB-42R 5′-GCTGAAGAACCCAAGCAGAC-3′
1AB-42F 5′-GGTCCCGAATTAAGGATGGT-3′
1AB-44R 5′-CTTCGTCGACGTCAGACTTG-3′
1AB-44F 5′-AGACCAAGCCCCGAATCTAT-3′
1AB-49R 5′-ATCGAATTTGTAGCCAACCG-3′
1AB-49F 5′-AAGAAGCGCAAGCTGATTGT-3′
1AB-51R 5′-TCATGAATCTGGTGCTTTGC-3′
1AB-51F 5′-GATCGAGCGCAATCGTTATT-3′
1B-4R 5′-AGTCCATCGACGTTGTTTCC-3′
1B-4F 5′-CCCGCCATCATCAACTATCT-3′
1B-6R 5′-ACGTTTTCGTTCAGATTGGC-3′
1B-6F 5′-GTTACTCCGGGTCATGTGCT-3′
1B-9R 5′-GTTCTGCGACATGCTGTGAT-3′
1B-9F 5′-ACCTAAGCTGAAACGGGGAT-3′
1B-10R 5′-CAGACCTTCTCCAGCGTT-3′
1B-10F 5′-AGTACCAGCAGGGCAAAGAA-3′
1B-12R 5′-CACAAAAGCGCATCTTCAAA-3′
1B-12F 5′-AATGTGCCAAAAGTTCCTGC-3′
1B-13R 5′-ACCGGTCTTTTCCTCCAGAT-3′
1B-13F 5′-ACTTCCGTGACCCGTACAAG-3′
1B-14R 5′-GTGGTGCAACTTAGGCCACT-3′
1B-14F 5′-AGCTGAGCTGAAGAAGCTGG-3′
1B-17R 5′-TGTTGTCCGTAACATCCGAA-3′
1B-17F 5′-CGATGAGGAGCAGTTCAACA-3′
1B-20R 5′-TACCGACGACCTTCAACTCC-3′
1B-20F 5′-CAGTCCGCTGATGAGAACAA-3′
1B-21R 5′-GGTACTTGCCATCGTCCTGT-3′
1B-21F 5′-TGGACGCTACTATCCGGAAC-3′
1B-22R 5′-CTGCGGTGTGGGTATCTTCT-3′
1B-22F 5′-TGGCGTTAACTCGACCTTCT-3′
1B-23R 5′-CCTGCTTCTCACGGTTCTTC-3′
1B-23F 5′-ATGAAATCCGCAAGGTTCTG-3′
1B-24R 5′-GTGCTGGTCGTTGTAGGG-3′
1B-24F 5′-CTGGCCAAATCGTACCAAGT-3′
1B-25R 5′-ATGCTGCTGCTGTCCCTAGT-3′
1B-25F 5′-TTAAAACCGTGACACCGTGA-3′
1B-27R 5′-AACCACTTCATTGCGTCCTC-3′
1B-27F 5′-CGTCGATCGGTATCTACGGT-3′
1B-29R 5′-ATCAACTTCCTCAACGGTGG-3′
1B-29F 5′-CCGAGAAGGACGAGTTTGAG-3′
1B-32R 5′-GTCTGCTTGGTTGGAATGGT-3′
1B-32F 5′-CACGATATTGTGCTGGTTGG-3′
1B-53R 5′-TCTCGTAGGTGGCCTTATCG-3′
1B-53F 5′-CGCAAAAGACCCAGAAGAAG-3′
5B-5R 5′-ATCGTTGGCGTAGTTGCTCT-3′
5B-5F 5′-GTCAACCCCTGGTCAAAATG-3′
5B-7R 5′-CGACGACAGAATGTACCCCT-3′
5B-7F 5′-AAGAGTATCTGGCGGAGGGT-3′
CxActin-629F 5′-TGCGTGACATCAAGGAGAAG-3′
CxActin-893R 5′-GTGTTGGCGTACAGGTCCTT-3′

[a] Primer names correspond to the clone numbers in Table 1.

different genes: troponinC, isocitrate dehydrogenase, allergen, cytochrome b5, chymotrypsinogen, apolipophorins, tryptase gamma, carboxylesterase, prolycarboxypeptidase, imaginal disc growth factor, aldehyde dehydrogenase, tropomyosin-1, chitotriosidase-1, heat shock protein 70B2, and inorganic phosphate cotransporter. We also found 11 clones encoding six hypothetical and five conserved hypothetical proteins. In addition, we found 15 clones that encoded for *Cx. quinquefasciatus* cytosolic ribosomal subunit, 60S ribosomal proteins, and one mitochondrial large subunit ribosomal RNA (Table 1).

Magnesium Effects on RNA Relative Expression Levels of Different Genes in Larval *Cx. quinquefasciatus*. qPCR analyses were conducted to examine the differential transcription of genes identified in response to magnesium in *Cx. quinquefasciatus* larvae. According to our qPCR data, most genes present in the subtracted cDNA library were upregulated (Fig. 1A). Compared with the control, the RNA relative expression level of apolipophorins (1AB-25), prolylcarboxypeptidase (1AB-51), imaginal disc growth factor (1B-4), and tryptase (1AB-36) increased significantly 10-fold for 1-h Mg^{2+} treatment of *Cx. quinquefasciatus* larvae (Fig. 1A; Supplemental Table 3A [available online only]). Troponin (1AB-3, 1AB-16, and 1AB-53), allergen (1AB-14, 1AB-35, 1B-9), cytochrome b5 (11AB-18), chymotrypsinogen (1AB-19, and 1B-6), carboxylesterase (1AB-42), aldehyde dehydrogenase (1B-8, and 1B-17), isocitrate dehydrogenase (1B-10), tropomyosin-1 (1B-20), chitotriosidase-1 (1B-24), and heat shock protein 70 B2 (1B-29) increased ≈2- to 6-fold for the 1-h Mg^{2+} treatment of *Cx. quinquefasciatus* larvae (Fig. 1A). Inorganic phosphate cotransporter (5B-7) found in the reverse subtractive cDNA library was downregulated for the 1-h Mg^{2+} treatment of *Cx. quinquefasciatus* larvae (Fig. 1A). All of these genes identified in response to Mg^{2+} exposure for 1 h were significantly different, when compared with the control (Supplemental Table 3 [available online only]). A >9-fold increase in the transcription of genes of hypothetical proteins (e.g., 1AB-4, 1AB-15, and 1AB-44) and conserved hypothetical protein mRNA (1B-14 and 1B-21) was found after the 1-h Mg^{2+} treatment of *Cx. quinquefasciatus*, which was significantly different from the control (Fig. 1B, Table 1; Supplemental Table 3B [available online only]).

In addition, RNA expression levels of several ribosomal genes were modulated and upregulated more than two times. The qPCR analysis further substantiated the differential transcription of the mitochondrial large subunit ribosomal gene (GenBank accession number: gb DQ401444.1) in response to Mg^{2+} exposure (Fig. 1C; Supplemental Table 3C [available online only]).

We also conducted a time course study of magnesium exposure on the relative RNA expression levels of several different genes in larval *Cx. quinquefasciatus* to better understand early gene response and regulation. RNA expression levels of many genes were modulated, some showing a >5-fold increase in expression during the Mg^{2+} exposure. For example, the imaginal disc growth factor gene was significantly upregulated

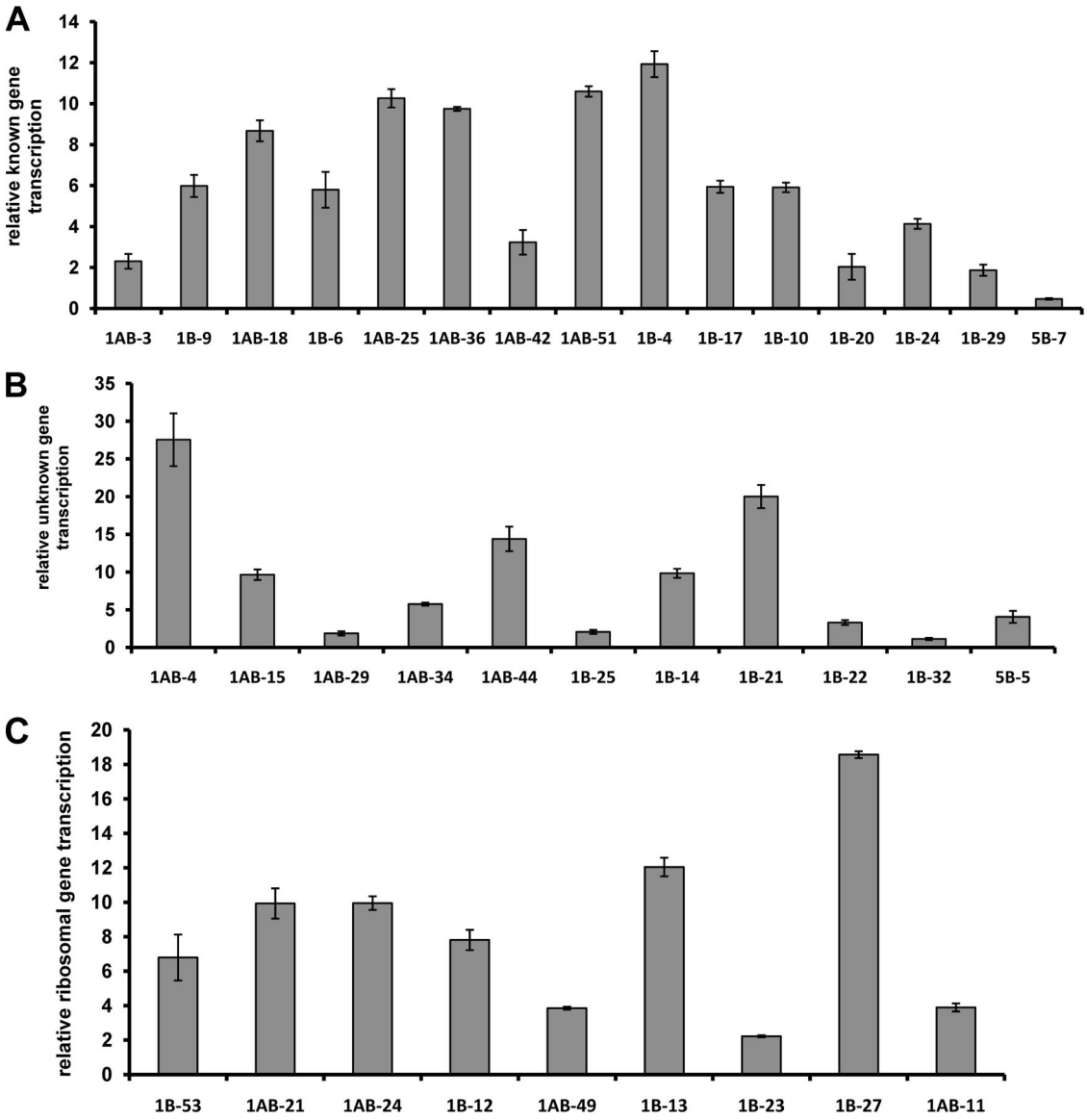

Fig. 1. The qPCR showed the relative gene transcription (fold increase) after magnesium treatment for 1 h compared with the control. (A) Known genes (Table 1) differentially transcribed after magnesium treatment for 1 h compared with the control (nonmagnesium treatment). (B) Unknown/Conserved genes (Table 1) differentially transcribed after magnesium treatment for 1 h compared with the control. (C) Ribosomal genes (Table 1) differentially transcribed after magnesium treatment for 1 h compared with the control.

>10-fold after 1 h of magnesium exposure (Fig. 2A; Supplemental Table 4A and G [available online only]). This same gene, however, was downregulated after 4 h of magnesium exposure (Fig. 2A). According to our qPCR data, relative gene transcription of carboxylesterase, chitotriosidase, and apolipophorin have similar patterns (i.e., upregulated at both 15 min and 1 h of Mg^{2+} exposure, and downregulated after 2 and 4 h of Mg^{2+} exposure) (Fig. 2B, C, and F; Supplemental Table 4B, C, F, and G [available online only]). For chymotrypsinogen, relative gene transcription was downregulated after 4 h of Mg^{2+} exposure (Fig. 2D; Supplemental Table 4D and G [available online only]). Although the relative gene transcription of cytochrome b5 was upregulated throughout the time course of Mg^{2+} exposure, it reached ≈8.7-fold more than the control after 1-h Mg^{2+} exposure and was significantly different (Fig. 2E; Supplemental Table 4E and G [available online only]).

Discussion

Genes upregulated in response to elevated magnesium concentrations could be involved in salt regula-

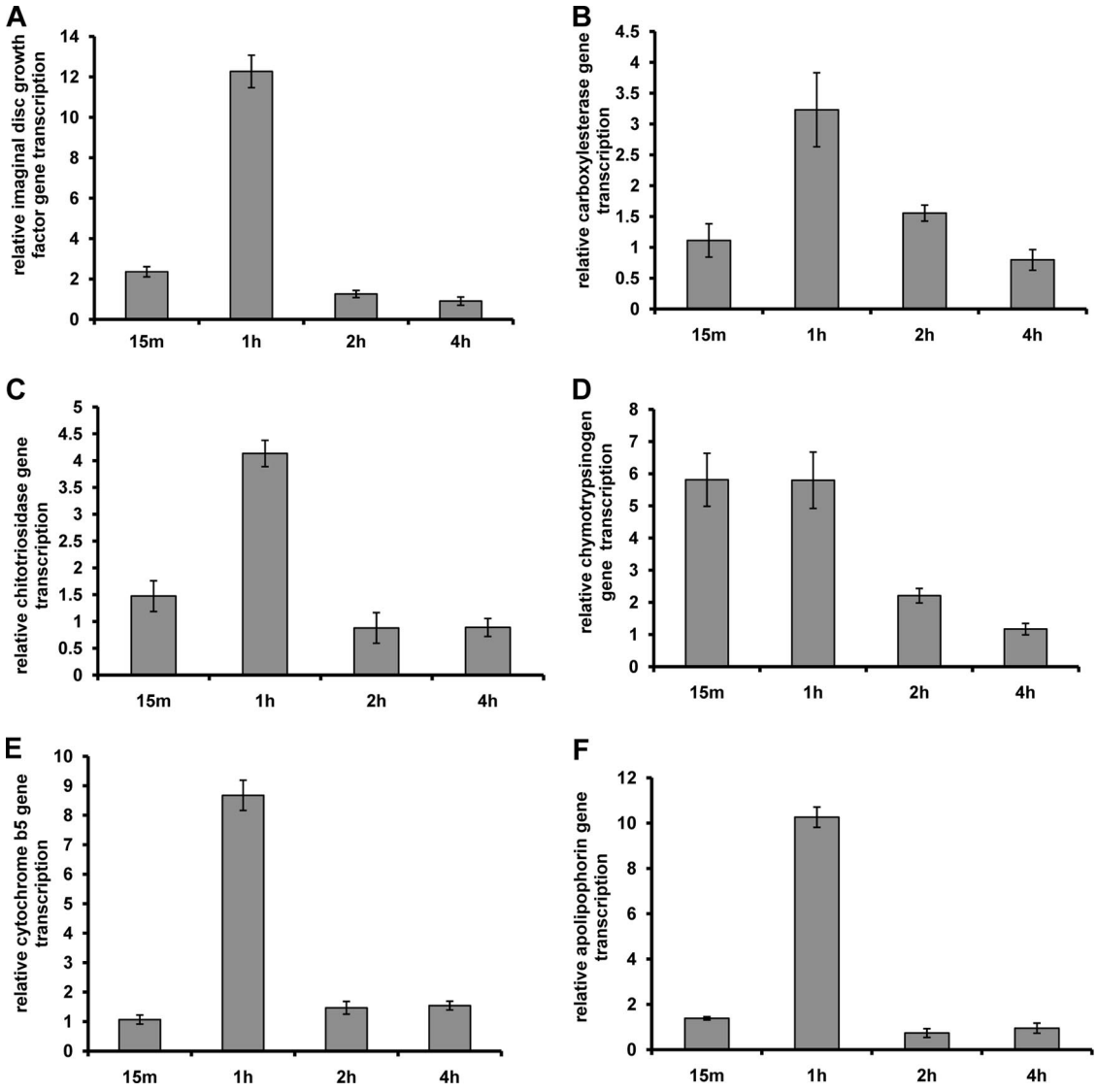

Fig. 2. The qPCR showed the relative gene transcription (fold increase) during the time course (15 min, 1 h, 2 h, and 4 h). (A) Relative imaginal disc growth factor gene (1B-4) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h. (B) Relative carboxylesterase gene (1AB-42) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h. (C) Relative chitotriosidase gene (1B-24) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h. (D) Relative chymotrypsinogen gene (1B-6) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h. (E) Relative cytochrome b5 gene (1AB-18) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h. (F) Relative apolipophorin gene (1AB-25) transcription (fold increase) after magnesium treatment compared with the control at 15 min, 1 h, 2 h, and 4 h.

tion and other physiological functions in mosquitoes, but might also serve to enhance baculovirus infection in the midgut. It has been reported that magnesium is crucial for transmission of baculovirus CuniNPV in *Cx. nigripalpus* and *Cx. quinquefasciatus* (Becnel et al. 2001). It was also shown that calcium is a potent inhibitor of transmission of CuniNPV even in the presence of magnesium. Our current study shows that magnesium levels that are sufficient for CuniNPV transmission can alter the gene transcription of larval *Cx. quinquefasciatus*. There are 15 known genes and 11 unknown genes that are up/downregulated during the Mg^{2+} exposure in *Culex* larvae (Table 1, Figs. 1 and 2). Specifically, genes that are overexpressed in the midgut, where baculovirus entry and replication occur, may play a role in enhanced viral transmission.

Carboxylesterases are ubiquitous enzymes and play diverse metabolic roles in mammalian organisms, insects, and plants, and in the detoxification of a broad range of drugs and xenobiotics (Fonnum et al. 1985;

Potter and Wadkins 2006; Cui et al. 2007a, 2007b; Cummins et al. 2007; Holmes et al. 2009). One study showed that the activities of carboxylesterases in response to three insecticides (i.e., malathion, propoxur, and permethrin) differed by mosquito species: high activities of carboxylesterases were detected in *Culex tritaeniorhynchus* (Giles), but not *Culex gelidus* (Theobald) (Karunaratne and Hemingway 2000). In a previous study, the expression of carboxylesterase genes in the mosquito *Culex pipiens* (L.) was important in resistance to organophosphate insecticides (Cui et al. 2007a). In *Ae. aegypti* larvae, induction of several cytochrome P450 genes and a carboxylesterase gene by exposure to low doses of various xenobiotics (e.g., insecticide, copper) increased larval tolerance to insecticides (Poupardin et al. 2008). These authors suggested that exposure of mosquito larvae in natural sites to low levels of xenobiotics affects tolerance to insecticides, possibly through cross-induction of certain detoxification genes. In this study, there was a significant 3.2-fold increase in the transcription of a carboxylesterase gene induced by 1-h magnesium exposure. Induction of a carboxylesterase gene by magnesium may also increase the tolerance of larval mosquitoes to pathogens by some unknown mechanism and could have an impact on virus infection in the midgut of *Culex* larvae.

Recently, mammalian chitinases have attracted much attention, because chitotriosidase plays a key role in the pathogenesis of asthma and in plasma of Gaucher patients (Hollak et al. 1994; Boot et al. 1995; Renkema et al. 1995, 1998; Zhu et al. 2004). Chitotriosidase secreted by activated human macrophages is involved in the defense against chitin-containing pathogens such as fungi, nematodes, and insects (Grosso et al. 2004, Gordon-Thomson et al. 2009). Studies by Di Luca et al. (2006, 2007) showed that human chitotriosidases from different sources helped *Plasmodium falciparum* (Welch) enter the *Anopheles* midgut presumably through degradation of the peritrophic matrix (PM). According to our data, chitotriosidase gene transcription was slightly upregulated after 15-min (1.47-fold) or 1-h (4.13-fold) Mg^{2+} exposure compared with the control and downregulated after 2- and 4-h Mg^{2+} exposure (Fig. 2C; Supplemental Table 4C and G [available online only]). Virions of CuniNPV must pass through the larval PM to attach to and enter midgut cells, and this occurs during the first hours of exposure. Degradation of PM in larval mosquitoes by chitotriosidase produced in response to magnesium exposure could serve to enhance infectivity by CuniNPV in the midgut of *Culex* larvae.

Chymotrypsinogen, a precursor of the digestive enzyme chymotrypsin, is converted to chymotrypsin by trypsin. Chymotrypsinogen is involved in the solubilization and activation of insecticidal protein toxins produced by *Bacillus thuringiensis* (Zhu et al. 1997). In this study, the transcription of a chymotrypsinogen gene was upregulated (5.8-fold) after 15 min (Fig. 2D). The virions of CuniNPV are encapsulated by an occlusion body protein with 4–8 virions per occlusion body (OB). Once OBs are ingested by mosquito larvae, the occlusion body protein must be solubilized to release virions before crossing the PM and attachment to microvilli of midgut cells (Becnel et al. 2001). A host response to Mg^{2+} exposure that increases the production of digestive enzymes such as chymotrypsinogen could mediate the release of CuniNPV virions from OBs and plays a role in enhancing virus infection.

Apolipophorins contain the major component of lipophorin, which mediates transport for various types of lipids in hemolymph and acts by forming lipoprotein particles that bind lipoproteins and lipids. There are two mature apolipophorins: apolipophorin-I and apolipophorin-II (Marinotti et al. 2006). In the insect hemolymph, the complex of low-density lipophorin, high-density lipophorin, and apolipophorin III provides a reusable lipid shuttle for flight muscle energy supply (Surholt et al. 1992). An apolipophorins gene was expressed in fat body tissues throughout development in *Anopheles gambiae* (Marinotti et al. 2006). In a previous study, the expression of apolipophorins in *Ae. aegypti* was induced by blood feeding, and lipophorin was synthesized in the fat body (van Heusden et al. 1998). Our data show that an apolipophorins gene increased >10-fold after 1-h Mg^{2+} exposure (Fig. 2F). There were also significant differences in the transcription of the apolipophorins gene between magnesium exposure at 1 h and the control in *Culex* larvae (Supplemental Table 4F and G [available online only]). The transcription of apolipophorins in larval *Cx. quinquefasciatus* was induced by Mg^{2+} exposure, which could elevate lipophorin synthesis in the fat body and change the lipid structure of the cell membrane.

Some hypothetical proteins were highly upregulated in response to Mg^{2+} exposure (1AB-15, 1AB-4, 1B-14, 1B-21, and 1AB-44). According to ExPASy ("DAS," Transmembrane Prediction Server), all of these genes have potential transmembrane segments, but their possible role in salt regulation and/or baculovirus transmission is unknown. The imaginal disc growth factor (1B-4) is also highly upregulated, but how this protein functions in mosquitoes is unknown. Notably, some MRGs found in previous studies with Diptera such as AEIMUCI (*Ae. aegypti* intestinal mucin) and CTTUBI (*Chiromomus tentans* α-tubulin) were not identified in this study (Beaty et al. 2002). Sublethal concentrations of toxic metals, such as copper and calmium, induced expression of these two proteins, which could enhance survivorship of aquatic arthropods in polluted environments. Because magnesium is not normally toxic to mosquito larvae, the lack of overexpression of homologues is not unexpected.

The current study suggests that certain genes (e.g., chymotrypsinogen, chitotriosidase, carboxylesterases, apolipophorin) expressed in response to magnesium exposure may play a role in the interaction of *Cx. quinquefasciatus* larvae and infection by the baculoviruses CuniNPV. Identification of these genes is the first step in determining the mechanistic role of magnesium in transmission of baculovirus in mosquitoes, and also provides basic information on genes that may

be involved in other physiological responses in mosquitoes.

Acknowledgments

We thank Drs. S. M. Valles (United States Department of Agriculture–Agricultural Research Service) and L. Zhou (University of Florida) for critical reviews of the manuscript. We also thank Neil Sanscrainte (United States Department of Agriculture–Agricultural Research Service) for helpful support. This work was supported by a grant from the Deployed War-Fighter Protection Research Program funded by the United States Department of Defense through the Armed Forces Pest Management Board.

References Cited

Beaty, B. J., R. S. Mackie, K. S. Mattingly, J. O. Carlson, and A. Rayms-Keller. 2002. The midgut epithelium of aquatic arthropods: a critical target organ in environmental toxicology. Environ. Health Perspect. 110(Suppl. 6): 911–914.

Becnel, J. J. 2006. Prospects for the mosquito baculovirus CuniNPV as a tool for mosquito control. J. Am. Mosq. Control Assoc. 22: 523–526.

Becnel, J., S. White, B. Moser, T. Fukuda, M. Rotstein, A. Undeen, and A. Cockburn. 2001. Epizootiology and transmission of a newly discovered baculovirus from the mosquitoes *Culex nigripalpus* and *C. quinquefasciatus.* J. Gen. Virol. 82: 275–282.

Boot, R. G., G. H. Renkema, A. Strijland, A. J. van Zonneveld, and J. M. Aerts. 1995. Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. J. Biol. Chem. 270: 26252–26256.

Cui, F., M. Weill, A. Berthomieu, M. Raymond, and C. L. Qiao. 2007a. Characterization of novel esterases in insecticide-resistant mosquitoes. Insect Biochem. Mol. Biol. 37: 1131–1137.

Cui, F., H. Qu, J. Cong, X. L. Liu, and C. L. Qiao. 2007b. Do mosquitoes acquire organophosphate resistance by functional changes in carboxylesterases? FASEB J. 21: 3584–3591.

Cummins, I., M. Landrum, P. G. Steel, and R. Edwards. 2007. Structure activity studies with xenobiotic substrates using carboxylesterases isolated from *Arabidopsis thaliana.* Phytochemistry 68: 811–818.

Di Luca, M., R. Romi, F. Severini, L. Toma, M. Musumeci, A. M. Fausto, M. Mazzini, G. Gambellini, and S. Musumeci. 2006. Human chitotriosidase helps *Plasmodium falciparum* in the *Anopheles* midgut. J. Vector Borne Dis. 43: 144–146.

Di Luca, M., R. Romi, F. Severini, L. Toma, M. Musumeci, A. M. Fausto, M. Mazzini, G. Gambellini, and S. Musumeci. 2007. High levels of human chitotriosidase hinder the formation of peritrophic membrane in anopheline vectors. Parasitol. Res. 100: 1033–1039.

Fonnum, F., S. H. Sterri, P. Aas, and H. Johnsen. 1985. Carboxylesterases, importance for detoxification of organophosphorus anticholinesterases and trichothecenes. Fundam. Appl. Toxicol. 5: S29–S38.

Gerberg, E. J., D. R. Barnard, and R. A. Ward. 1994. Manual for Mosquito Rearing and Experimental Techniques, Bulletin No. 5. American Mosquito Control Association, Lake Charles, LA.

Gordon-Thomson, C., A. Kumari, L. Tomkins, P. Holford, J. T. Djordjevic, L. C. Wright, T. C. Sorrell, and G. P. Moore. 2009. Chitotriosidase and gene therapy for fungal infections. Cell Mol. Life Sci. 66: 1116–1125.

Grosso, S., M. A. Margollicci, E. Bargagli, Q. R. Buccoliero, A. Perrone, D. Galimberti, G. Morgese, P. Balestri, and P. Rottoli. 2004. Serum levels of chitotriosidase as a marker of disease activity and clinical stage in sarcoidosis. Scand. J. Clin. Lab. Invest. 64: 57–62.

Hollak, C. E., S. van Weely, M. H. van Oers, and J. M. Aerts. 1994. Marked elevation of plasma chitotriosidase activity: a novel hallmark of Gaucher disease. J. Clin. Invest. 93: 1288–1292.

Holmes, R. S., J. P. Glenn, J. L. VandeBerg, and L. A. Cox. 2009. Baboon carboxylesterases 1 and 2: sequences, structures and phylogenetic relationships with human and other primate carboxylesterases. J. Med. Primatol. 38: 27–38.

Karunaratne, S. H., and J. Hemingway. 2000. Insecticide resistance spectra and resistance mechanisms in populations of Japanese encephalitis vector mosquitoes, *Culex tritaeniorhynchus* and *Cx. gelidus,* in Sri Lanka. Med. Vet. Entomol. 14: 430–436.

Marinotti, O., L. Capurro Mde, X. Nirmala, E. Calvo, and A. A. James. 2006. Structure and expression of the lipophorin-encoding gene of the malaria vector, *Anopheles gambiae.* Comp. Biochem. Physiol. B Biochem. Mol. Biol. 144: 101–109.

Patel, M., and H. Sive. 2001. PCR-based subtractive cDNA cloning. Curr. Protoc. Mol. Biol. Chapter 25: Unit 25B 2.

Potter, P. M., and R. M. Wadkins. 2006. Carboxylesterases: detoxifying enzymes and targets for drug therapy. Curr. Med. Chem. 13: 1045–1054.

Poupardin, R., S. Reynaud, C. Strode, H. Ranson, J. Vontas, and J. P. David. 2008. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti:* impact on larval tolerance to chemical insecticides. Insect Biochem. Mol. Biol. 38: 540–551.

Rayms-Keller, A., M. McGaw, C. Oray, J. O. Carlson, and B. J. Beaty. 2000. Molecular cloning and characterization of a metal responsive *Aedes aegypti* intestinal mucin cDNA. Insect Mol. Biol. 9: 419–426.

Renkema, G. H., R. G. Boot, A. O. Muijsers, W. E. Donker-Koopman, and J. M. Aerts. 1995. Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. J. Biol. Chem. 270: 2198–2202.

Renkema, G. H., R. G. Boot, F. L. Au, W. E. Donker-Koopman, A. Strijland, A. O. Muijsers, M. Hrebicek, and J. M. Aerts. 1998. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. Eur. J. Biochem. 251: 504–509.

Surholt, B., J. M. Van Doorn, J. Goldberg, and D. J. Van der Horst. 1992. Insect lipophorin conversions: compositional analysis of high- and low-density lipophorin of *Acherontia atropos* and *Locusta migratoria.* Biol. Chem. Hoppe Seyler 373: 13–20.

van Heusden, M. C., F. Thompson, and J. Dennis. 1998. Biosynthesis of *Aedes aegypti* lipophorin and gene expression of its apolipoproteins. Insect Biochem. Mol. Biol. 28: 733–738.

Zhao, L., J. W. Pridgeon, J. J. Becnel, G. G. Clark, and K. J. Linthicum. 2008. Cytochrome *c* gene and protein expression: developmental regulation, environmental response, and pesticide sensitivity in *Aedes aegypti.* J. Med. Entomol. 45: 401–408.

Zhao, L., J. W. Pridgeon, J. J. Becnel, G. G. Clark, and K. J. Linthicum. 2009. Identification of genes differentially

expressed during heat shock treatment in *Aedes aegypti.* J. Med. Entomol. 46: 490–495.

Zhu, Y. C., B. Oppert, K. J. Kramer, W. H. McGaughey, and A. K. Dowdy. 1997. cDNAs for a chymotrypsinogen-like protein from two strains of *Plodia interpunctella.* Insect Biochem. Mol. Biol. 27: 1027–1037.

Zhu, Z., T. Zheng, R. J. Homer, Y. K. Kim, N. Y. Chen, L. Cohn, Q. Hamid, and J. A. Elias. 2004. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 304: 1678–1682.

Received 5 February 2010; accepted 30 June 2010.